INHIBITORS OF CRYSTALLIZATION IN A SOLID DISPERSION

Technical Field of the Invention

The instant invention relates to the fields of pharmaceutical and organic chemistry, and provides novel solid dispersion pharmaceutical formulations which demonstrate an inhibition of crystallization.

Background of the Invention

One measure of the potential usefulness of an oral dosage form of a pharmaceutical agent is the bioavailability observed after oral administration of the dosage form. Various factors can affect the bioavailability of a drug when administered orally. These factors include aqueous solubility, drug absorption throughout the gastrointestinal tract, dosage strength, and first pass effect. Aqueous solubility is one of the most important of these factors. When a drug has poor aqueous solubility, attempts are often made to identify salts or other derivatives of the drug which have improved aqueous solubility. When a salt or other derivative of the drug is

identified which has good aqueous solubility, it is generally accepted that an aqueous solution formulation of this salt or derivative will provide the optimum oral bioavailability. The bioavailability of the aqueous oral solution formulation of a drug is then generally used as the standard or ideal bioavailability against which other oral dosage forms are measured.

For a variety of reasons, including patient compliance and taste masking, a solid dosage form, such as a capsule or tablet, is usually preferred over a liquid dosage form. However, oral solid dosage forms of a drug generally provide a lower bioavailability than oral solutions of the drug. One goal of the development of a suitable solid dosage form is to obtain a bioavailability of the drug that is as close as possible to the ideal bioavailability demonstrated by the oral aqueous solution formulation of the drug.

An alternative dosage form is a solid dispersion. The term solid dispersion refers to the dispersion of one or more active ingredients in an inert carrier or matrix at solid state prepared by the melting (or fusion), solvent, or melting-solvent methods. (Chiou and Riegelman, *Journal of Pharmaceutical Sciences*, *60*, 1281 (1971)). The dispersion of a drug or drugs in a solid diluent by

mechanical mixing is not included in this category. Solid dispersions may also be called solid-state dispersions.

Retroviral protease inhibiting compounds are useful for inhibiting HIV proteases *in vitro* and *in vivo*, and are useful for inhibiting HIV (human immunodeficiency virus) infections and for treating AIDS (acquired immunodeficiency syndrome). HIV protease inhibiting compounds typically are characterized by having poor oral bioavailability. Examples of HIV protease inhibiting compounds include

2S,3S,5S)-5-(N-(N-((N-methyl-N-((2-isopropyl-4-thiazolyl)methyl)amino)carbonyl)L-valinyl)amino-2-(N-((5-thiazolyl)methoxy-carbonyl)-amino)-amino-1,6-diphenyl-3-hydroxyhexane (ritonavir);

(2S, 3S, 5S)-2-(2,6-Dimethylphenoxyacetyl) amino-3-hydroxy-5-[2S-(1-tetrahydro-pyrimid-2-onyl)-3-methyl butanoyl]-amino-1,6-diphenylhexane (ABT-378);

N-(2(R)-hydroxy-1 (S)-indanyl)-2(R)-phenylmethyl -4(S)-hydroxy-5-(1-(4-(3-pyridylmethyl)-2(S)-N'-(t-butylcar boxamido)-piperazinyl))-pentaneamide (indinavir);

N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4aS,8aS) -isoquinoline-3(S)-carboxamide (saquinavir);

5(S)-Boc-amino-4(S)-hydroxy-6-phenyl-2(R)-phenylmethylhexanoyl-(L)-Val-(L)-Phe-morpholin-4-ylamide;

1 -Naphthoxyacetyl-beta-methylthio-Ala-(2S, 3S)-3-amino-2-hydroxy-4-butanoyl 1,3-thiazolidine-4-t-butylamide;

5-isoquinolinoxyacetyl-beta-methylthio-Ala-(2S,3S)-3-amino-2-hydroxy-4-butanoyl-1,3-thiazolidine-4-t-butylamide;

[1S-[1R-(R-),2S*])-N^1 [3-[[[(1,1 -dimethylethyl)amino]carbonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl)propyl]-2-[(2-quinolinylcarbonyl)amino]-butanediamide;

VX-478; DMP-323; DMP-450; AG1343(nelfinavir);

BMS 186,318; SC-55389a; BILA 1096 BS; and U-140690, or combinations thereof.

While some drugs would be expected to have good solubility in organic solvents, it would not necessarily follow that oral administration of such a solution would give good bioavailability for the drug.

Polyethylene glycol (PEG) solid dispersion formulations are generally known to improve the dissolution and bioavailability of many compounds. However, Aungst *et al.* has recently demonstrated that this was unable to improve the bioavailability of an HIV protease inhibitor with a cyclic urea structural backbone, called DMP 323

(Aungst *et al.*, *International Journal of Pharmaceutics*, *156*, 79 (1997)).

In addition, some drugs tend to form crystals when placed in solution, which can be problematic during formulation.

Polyvinylpyrrolidone (PVP) is known to inhibit crystallization of drugs (Yohioka, M. et al., J. Pharm. Sci., 84, 983, 1995). However, prior to the instant invention, the incorporation of PVP into a second polymer matrix, such as polyethylene glycol, has never been established.

U.S. 4,610,875 teaches a process for the preparation of a stable pharmaceutical dipyridamole composition containing PVP.

U.S. 4,769,236 teaches a process for the preparation of a stable pharmaceutical composition with a high dissolution rate in the gastrointestinal tract containing PVP, wherein the pharmaceutical agent is hydroflumethiazide, dipyridamole, hydrochlorothiazide, cyclothiazide, cyclopenthiazide, polythiazide, methyldopa, spironolactone, quinidine, cyanidol, metronidazole, ibuprofen, naproxen, erythromycin, glaphenin, furosemide, suloctidil, nitrofurantoin, indomethacin, flavoxate,

phenobarbitol, cyclandelate, ketoprofen, natridrofuryl, or triamterene.

Thus, it would be a significant contribution to the art to provide a stable solid dispersion pharmaceutical formulation which demonstrates a lack of crystallization.

Summary of the Invention

The instant invention provides a stable solid dispersion pharmaceutical formulation comprising a pharmaceutical compound, a water soluble carrier, such as polyehtylene glycol (PEG), and a crystallization inhibitor, such as polyvinylpyrrolidone (PVP) or hydroxypropylmethylcellulose (HPMC).

Also provided by the instant invention is a pharmaceutical composition comprising a stable solid dispersion as described above with additional pharmaceutically acceptable carriers, diluents, or excipients.

Additionally provided by the instant invention is a method for preparing a stable solid dispersion as described above.

The instant invention still further provides methods of treatment comprising administering an effective amount of a stable solid dispersion as described above to a mammal in need of such treatment.

Brief Description of the Figures

Figure 1 illustrates the PXD patterns showing that Amorphous ABT-538 can be isolated within PEG alone.

Figure 2 illustrates the PXD patterns showing that Amorphous ABT-538 can be isolated with a PVP/PEG matrix.

Figure 3 illustrates the DSC thermograms of PEG, ABT-538, a physical mixture of the two and a solid dispersion. The absence of ABT-538 melting in the dispersion confirms the above PXD data showing amorphous ABT-538 present in the dispersion.

Figure 4 illustrates the DSC thermograms of PVP/PEG, ABT-538, a physical mixture of the two and a solid dispersion. The absence of ABT-538 melting in the dispersion confirms the above PXD data showing amorphous ABT-538 present in the dispersion.

Figure 5 illustrates the effect of PEG or PVP on the crystallization rate of amorphous ritonavir. The heat of fusion was used to calculate percent crystallized. In the presence of PVP the crystallization rate is slower.

Figure 6 illustrates the inhibition of crystallization using PVP.

Figure 7 illustrates PXD patterns of ABT-538 dispersions with and without PVP stored at 50°C. The data

demonstrate the improved physical stability of amorphous ABT-538 on storage.

Figure 8 illustrates PXD patterns of fenofibrate dispersions with and without PVP.

Figure 9 illustrates PXD patterns of fenofibrate dispersions with and without PVP and PEG.

Figure 10 illustrates PXD patterns of fenofibrate dispersions with and without PEG.

Figure 11 illustrates PXD patterns of fenofibrate dispersions with and without 10% PVP and PEG.

Figure 12 illustrates PXD patterns of griseofulvin dispersions with and without PEG.

Figure 13 illustrates PXD patterns of griseofulvin dispersions with and without PEG and PVP.

Figure 14 illustrates PXD patterns of griseofulvin dispersions with and without PEG.

Figure 15 illustrates PXD patterns of griseofulvin dispersions with and without PEG and PVP.

Detailed Description of the Invention

This invention pertains to the preparation of solid dispersion systems for pharmaceuticals which demonstrate a lack of crystallization.

The invention involves dispersion in a hydrophilic matrix of pharmaceuticals which exhibit poor aqueous solubility. The intent of such a formulation is to improve the aqueous dissolution properties and ultimately achieve improved bioavailability. Typically, the intent of such systems is to generate a dispersion of amorphous (high energy) drug within the matrix. The presence of the high energy drug form usually improves the dissolution rate. However, these systems are not often physically stable. The drug can crystallize over time, causing the loss of the desired properties and reduced shelf-life. The current invention enhances the physical stability of such formulations, thereby making this type of formulation more feasible.

In the instant invention, PEG 8000 is used as the hydrophilic matrix. Also employed in this formulation is polyvinylpyrrolidone (PVP), which is an example of a hydrophilic, amorphous polymer, and is used to inhibit crystallization. Other hydrophilic, amorphous polymers

include hydroxypropylmethylcellulose (HPMC), or other pharmaceutically acceptable hydrophilic, amorphous polymers. Specifically, PVP PF 17 is used within the PEG matrix to inhibit the crystallization of the drug of interest. A range of 1%-95% (w/w) of PVP can be employed, with a range of 1%-15% (w/w) being preferred.

The benefits of incorporating PVP into the PEG matrix are two fold. Firstly, processing PVP can be difficult due to its hygroscopicity. Secondly, when PVP dissolves a viscous layer at the solid-liquid interface forms. This viscous region can hinder dissolution of the drug. Another benefit of adding PVP is an increase in amorphous volume of the polymer matrix where drugs may reside. Since polyethylene glycols tend to be highly crystalline, this increase in amorphous volume could be important for fast dissolution. PVP has the added advantage of having a high Tg, which imparts stabilization of amorphous regions by reducing mobility. Therefore, this invention affords the benefits of the PEG properties in a dispersion along with those of PVP.

A solid (molecular) dispersion comprising an HIV protease inhibiting compound may be prepared by dissolving or dispersing the HIV protease inhibiting compound in a sufficient amount of an organic solvent followed by

dispersion into a suitable water soluble carrier. Suitable organic solvents include pharmaceutically acceptable solvents such as methanol, ethanol, or other organic solvents in which the protease inhibitor is soluble. Suitable water soluble carriers include polymers such as polyethylene glycol (PEG), pluronics, pentaeythritol, pentaeythritol tetraacetate, polyoxyethylene stearates, poly-ε-caprolactone, and the like.

The organic solvent (preferably ethanol) may then be evaporated away, leaving the drug dispersed/dissolved in the molten matrix, which is then cooled. The solid matrix has the compound finely dispersed (molecular dispersion) in such a way that dissolution of the drug is maximized, thus improving the bioavailability of a drug exhibiting dissolution rate limited absorption. Ease of manufacturing is also an attribute to this type of formulation. Once the organic solvent is evaporated to yield a solid mass, the mass may be ground, sized, and optionally formulated into an appropriate delivery system. Thus, by improving the dissolution of a poorly water soluble drug, the drug in a suitable carrier may be filled into a gelatin capsule as a solid, or the matrix may potentially be compressed into a tablet.

The delivery system of the present invention results in increased solubility and bioavailability, and improved dissolution rate of the HIV protease inhibiting compound.

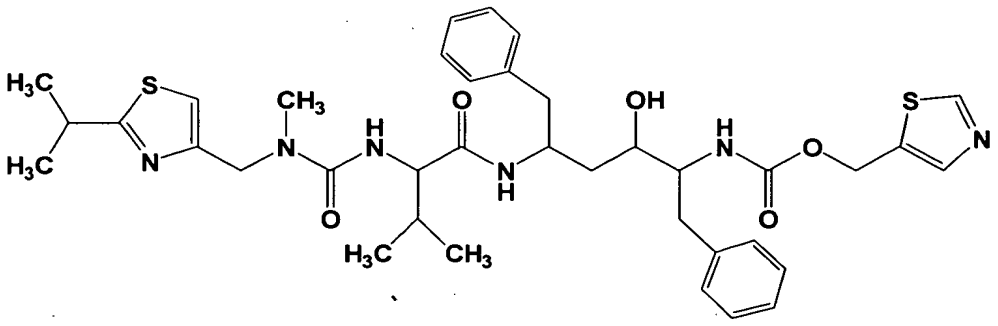
Other pharmaceutically-acceptable excipients may be added to the formulation prior to forming the desired final product. Suitable excipients include lactose, starch, magnesium stearate, or other pharmaceutically-acceptable fillers, diluents, lubricants, disintegrants, and the like, that might be needed to prepare a capsule or tablet.

The resulting composition comprising the pharmaceutical compound may be dosed directly for oral administration, diluted into an appropriate vehicle for oral administration, filled into capsules, or made into tablets for oral administration, or delivered by some other means obvious to those skilled in the art. The composition can be used to improve the oral bioavailability and solubility of said HIV protease inhibiting compound.

Total daily dosing of the pharmaceutical compound may be administered to a human in single or divided doses in amounts, for example, from 0.001 to 1000 mg/kg body weight daily, but more usually 0.1 to 50 mg/kg body weight daily. Dosage unit compositions may contain such amounts of

submultiples thereof to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, body weight, general health, sex, diet, time of administration, rate of excretion, drugs administered in combination and the severity of the particular disease undergoing therapy.

One type of pharmaceutical compound that may be employed in the practice of the present invention is an HIV protease inhibitor. An example of an HIV protease inhibitor is ABT-538 (ritonavir), the chemical structure of which is represented hereinbelow as a compound of formula I

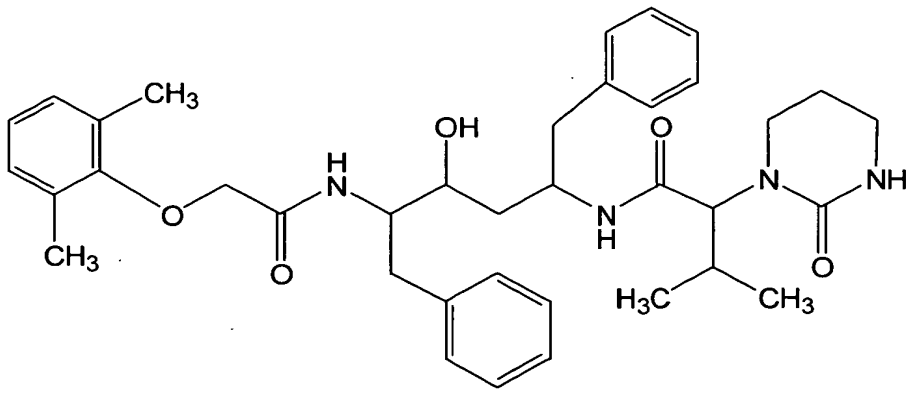


I

A compound of formula I is an HIV protease inhibitor marketed by Abbott Laboratories under the tradename Norvir®, with the common name ritonavir [(2S,3S,5S)-5-(N-(N-((N-

methyl-N-((2-isopropyl-4-thiazolyl)-methyl)amino)carbonyl)-L-valinyl)amino-2-(N-((5-thiazolyl)methoxy-carbonyl)-amino)-1,6-diphenyl-3-hydroxyhexane]. This and other compounds as well as methods for preparing same are disclosed in U.S. Patent Nos. 5,648,497 and 5,541,206, the disclosures of which are herein incorporated by reference.

Additional HIV protease inhibitors which may be formulated into a solid dispersion of the instant invention include compounds of formula II



II

A compound of formula II is known as ABT-378 ((2S,3S,5S)-2-(2,6-dimethylphenoxyacetyl)-amino-3-hydroxy-5-(2S-(1-tetrahydropyrimid-2-onyl)-3-methyl-butanoyl)amino-1,6-diphenylhexane). This and other compounds, as well as methods for preparing same, are identified in U.S. Patent No. 5,914,332, the disclosure of which is herein incorporated by reference.

Other types of pharmaceutical compounds which may be employed in the practice of the present invention include but are not limited to antibacterial agents, antifungal agents such as griseofulvin, chemotherapeutic agents, agents for treating hyperlipidemia such as fenoifibrate, and the like.

The following Examples are provided to further illustrate the present invention.

EXAMPLES

Equipment:

DSC

DSC measurements were made using a Mettler DSC 30 unit. Samples (4-7mg) were sealed in standard 40 μl aluminum crucibles with a single hole punched in the lids. An empty crucible of the same type was used as a reference.

X-ray Powder Diffraction Analysis

An X-ray powder diffraction (XPD) pattern was obtained with a Scintag® XDS 2000 θ/θ diffraction system equipped with a 2 kW normal focus X-ray tube and a liquid nitrogen cooled germanium solid state detector.

Isothermal Calorimetry (TAM)

The recrystallization reactions of 30% ABT-538 in PEG or PEG:PVP (95:5) solid dispersions were monitored via isothermal calorimetry (Thermometric 2277 Calorimeter) at 40 °C. Since crystallization is an exothermic process, a positive power output indicates

crystallization. The magnitude of the power output at any time is proportional to the rate of crystallization. XPD was used to confirm crystallization.

HPLC

The potency values of all the dispersions as well as the dissolution sample concentrations were determined via HPLC.

The effect of PVP on the crystallization rate of the drug in each dispersion system (drug with polymer) was investigated with the appropriate experimental technique. The results of these studies are provided in Figures 1-15.

Three pharmaceuticals of different properties were employed to demonstrate the general applicability of the instant invention. These compounds are identified in Table 1 below:

Table 1

Model Compounds

Property/Compound	ABT-538	Fenofibrate	Griseofulvin
MW (g/mole)	720.96	360.84	352.77
T_m (°C)	124	79	218.13
T_g (°C)	45.8	-21.7	91

Example 1

Dispersion Preparations

A. Ritonavir (ABT-538) Dispersion Preparation:

The samples were prepared by dissolving ABT-538 in a small volume of 200 proof ethanol in a 250 ml round bottom flask. The flask was vortexed and then placed in a water bath maintained at 75 °C. The PEG 8000 was added to the hot alcohol solution with continual swirling until the PEG melted. The flask was then attached to a rotary evaporator, immersed in the water bath (75 °C) under vacuum for 15 minutes to remove the ethanol. After the majority of ethanol had evaporated, the flask was immersed in an ice

bath for 15 minutes. The contents of the flask were then vacuum dried at room temperature overnight to remove residual alcohol. The dispersion was removed from the flask, gently ground, and sized to 40-100 mesh size. The drug loads used for these dispersions were 10, 20 and 30% w/w.

B. ABT-378 Dispersion Preparation:

The solid dispersion of 30% ABT-538 in 95:5 PEG8000:PVP was prepared by dissolving the ABT-538 and PVP 17 PF in a small volume of 200 proof ethanol in a 250 ml round bottom flask. The remainder of the process was as described above. A 30% ABT-538 dispersion in 85:15 PEG8000:PVP was also prepared similarly as were dispersions of 10 or 20% PVP 17PF in PEG 8000 without drug.

C. Fenofibrate Dispersion Preparation:

15% Fenofibrate in PEG 8000:

Both fenofibrate and PEG 8000 were sized to 40-100 mesh prior to mixing with a spatula on weighing paper. The mixture was then added to a 25 ml beaker and heated to 85°C in a water bath until the all the material had

melted. The molten solution was then poured onto a chilled X-ray sample holder to rapidly solidify the solution. The solid sample was immediately used to monitor the crystallization rate via X-ray powder diffraction.

15% Fenofibrate in 90:10 PEG 8000:PVP:

Fenofibrate (40-100 mesh) was added to the 90:10 PEG 8000:PVP control dispersion (see above) which was also sized to 40-100 mesh and mixed with spatula on a piece of weighing paper. The mixture was then processed as described above for the 15% fenofibrate dispersion in PEG 8000.

D. Griseofulvin Dispersion Preparation:

15% griseofulvin in PEG 8000:

Both griseofulvin and PEG 8000 were sized to 40-100 mesh prior to mixing on a weighing paper with a spatula. The sample was then added to an 4 ml stainless steel vessel which was sealed under a N_2 atmosphere. The vessel was then immersed into an oil bath maintained at 180°C. The sample was occasionally shaken to mix the molten contents. After 5 minutes the vessel was immersed into a

liquid N_2 bath for 30 minutes. The contents of the vessel were removed, gently ground and sized to 40-100 mesh.

15% griseofulvin in 80:20 PEG 8000:PVP:

This dispersion was prepared in a similar manner as describe above for the 15% griseofulvin in PEG 8000 dispersion using the 80:20 PEG8000:PVP control dispersion.

E. Results:

ABT-538:

Figure 1 shows the X-ray powder diffraction (XPD) pattern of ABT-538, processed PEG 8000, a physical mixture of the two components and the 30% solid dispersion. A similar plot is shown in Figure 2 with PVP incorporated into the matrix. It is apparent from these figures that ABT-538 is not crystalline within either matrix. Figure 3 shows the DSC thermograms of ABT-538, PEG8000, the 30% physical mixture and the dispersion. A similar plot is seen in Figure 4 for the PEG:PVP dispersion. The endotherm associated with drug melting can clearly be discerned from the other components. Thus, it is possible to follow the kinetics of ABT-538

crystallization via DSC measurements. Crystallization kinetics were determined by heating the samples to 85°C, holding them isothermally for predetermined times followed by heating through the melting transition temperature of ABT-538. The heats of fusion were determined and ratioed against the heat of fusion of the drug melting in the physical mixture, giving the fraction crystallized. The percent crystallized as a function of isothermal (85°C) hold time is shown in Figure 5. It is clear from this experiment that the presence of PVP within the matrix suppresses the crystallization rate of ABT-538.

The crystallization rate was also followed via the heat associated with crystallization of ABT-538 using a isothermal calorimetry. The shapes and magnitudes of the crystallization peaks in Figure 6 indicate that ABT-538 crystallizes more readily in the PEG matrix as compared to the PEG:PVP matrix. This stabilizing effect of PVP is also reflected in the times required for complete crystallization (time to reach baseline) which were <10 hours for PEG and >30 hours for PEG:PVP (95:5). These data support the previous DSC results.

An additional study was performed with a dispersion containing 15% PVP. The samples were held at 50°C (above

the T_g of ABT-538) and X-ray diffraction patterns were measured over time to monitor for the appearance of crystalline ABT-538. Figure 7 shows that in the presence of PVP, crystalline ABT-538 is not present after 272 hours, while in PEG8000 alone crystalline drug is detected at 233 hours (and before, data not shown).

Fenofibrate:

Figure 8 shows the XPD patterns of PEG 8000, fenofibrate, a 15% physical mixture and the 15% fenofibrate solid dispersion. The figure illustrates that the fenofibrate is X-ray-amorphous within the matrix. A similar plot with the XPD patterns for the 15% fenofibrate dispersion in a 90:10 PEG 8000:PVP matrix is presented in Figure 9. Again, the fenofibrate is amorphous. Upon storage at 25°C, the fenofibrate begins to crystallize in the PEG 8000 matrix within 1 hour (Figure 10). Additional crystallization follows upto 12 hours, when the experiment was terminated. In the presence of PVP (Figure 11), the fenofibrate does not crystallize in the timeframe of the experiment. This clearly demonstrates the inhibitory effects of PVP on crystallization within the PEG 8000 matrix.

Griseofulvin:

Similar XPD patterns for the griseofulvin dispersion in PEG 8000 and 80:20 PEG 8000:PVP matrices are shown in Figures 12 and 13, respectively. In both instances, amorphous griseofulvin is isolated within the respective matrices. The XPD rate of crystallization experiments show that after one hour at 25°C, griseofulvin begins to crystallize (Figure 14). However, in the presence of PVP (Figure 15), crystallization is not observed even after 15 hours under the same conditions. This again demonstrates the inhibitory effects of PVP amorphous drug crystallization within a PEG matrix.

E. Conclusions:

The data presented demonstrate that PVP incorporated within a hydrophilic matrix, such as PEG 8000, inhibits crystallization of drug molecules having varying physicochemical properties. Thus, the instant invention has a broad application to development of viable solid dispersion formulations where the high energy amorphous (non-crystalline) form of a drug is desired.

Example 2

Stability of Dispersion in Molten PEG 8000

The stability of the dispersion of ABT-538 in PEG 8000 in the molten state at 70 °C was examined. Individual approximately 5 mg quantities of the dispersion (aged for 6 weeks at room temperature) were placed in 4 ml glass vials. These vials, with the exception of the initial time point, were placed in a 70 °C oven which was sampled at pre-determined intervals, chilled in ice water and placed in the freezer until HPLC analysis. After all samples were collected, they were analyzed for ABT-538 content by HPLC. The HPLC system consisted of a Hitachi AS 4000 autosampler, SP 8800 ternary pump, Applied Biosystems 783 detector, and PE Nelson Data acquisition system. Other chromatographic details included a Regis Little Champ 5 cm C-18 column, a mobile phase consisting of an aqueous solution of 0.1% trifluoroacetic acid in 10 mM aqueous tetramethyl ammonium perchlorate (TMAP)/acetonitrile/methanol (55/40/5). The flow rate was 1 ml/minute, the wavelength of detection was 205 nm, and the injection volume was 100 μl. Standard curves of peak area of ABT-538 vs.

concentration in the range of interest were compared with experimentally obtained area counts.

Example 3

Protocol For Oral Bioavailability Studies

Dogs (beagle dogs, mixed sexes, weighing 7-14 kg) are fasted overnight prior to dosing, but are permitted water *ad libitum*. Each dog receives a 100 μg/kg subcutaneous dose of histamine approximately 30 minutes prior to dosing. Each dog receives a single solid dosage form corresponding to a 5 mg/kg dose of the drug. The dose is followed by approximately 10 milliliters of water. Blood samples are obtained from each animal prior to dosing and at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10 and 12 hours after drug administration. The plasma is separated from the red cells by centrifugation and frozen (- 30 °C) until analysis. The concentrations of parent drug is determined by reverse phase HPLC with low wavelength UV detection following liquid-liquid extraction of the plasma samples. The parent drug area under the curve is calculated by the trapezoidal method over the time course of the study. The absolute bioavailability of each test composition is calculated by comparing the area under the curve after oral dosing to

that obtained from a single intravenous dose. Each capsule or capsule composition is evaluated in a group containing at least six dogs. The values reported are averages for each group of dogs.

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